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Stable transformation of maize: the impact of feeder cells on protoplast growth and transformation efficiency

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Summary. The importance of cell culture conditions, including the use of feeder cells, on protoplast growth and transformation in maize (*Zea mays* L.) was investigated. Total GUS activity, measured two days after transformation, was five-fold higher in protoplasts cultured on feeder cells compared to those grown in the absence of feeder cells. Since the specific activity of GUS was only slightly higher in the transformed protoplasts plated over feeder cells, the stimulation in transient gene expression resulted mainly from the improved environment provided by the feeder system. For stable transformation, either PEG treatment or electroporation of protoplasts was used to introduce the *neo* gene. When PEG was used, over 85% of the putative transformants (resistant to kanamycin) contained the *neo* gene. The combination of PEG transformation and the optimized cell culture protocol using feeder cells enabled the selection of about 100 stably transformed lines per gFW of cells. Electroporation was less efficient.

Key words: *Zea mays* L. - Transformation - Protoplast - Kanamycin - ß-glucuronidase

Introduction

Plant genetic engineering has unprecedented potential for crop improvement. To obtain transgenic plants through protoplast treatment, it is necessary to merge two important technologies - cell culture and genetic transformation. In light of recent developments in the transformation techniques (Wang *et al.* 1988), the basic advantage of the protoplast system may be the generation of large quantities of uniformly transformed plant material and the possibility of initial screening for proper integration and expression at the level of callus tissue. However, the use of protoplasts requires efficient transformation methods and effective cell culture conditions.

Electroporation is commonly used for direct DNA transfer into monocot protoplasts (Fromm *et al.* 1986; Rhodes *et al.* 1988a; Hauptmann *et al.* 1988). However, chemical treatment with PEG may be less detrimental to protoplasts than electroporation (Vasil *et al.*1988) which may result in improved overall efficiency of stable transformation provided that protoplast culture conditions will not be the limiting factor.

One of the most important aspects of monocot protoplast growth into callus and plantlets is the use of feeder (or nurse) cells during the early stages of protoplast growth (Shillito *et al.* 1983; Kuang *et al.* 1984; Ludwig *et al.* 1985; Kamo *et al.* 1987; Somers *et al.* 1987; Rhodes *et al.* 1988a and b; Lee *et al.* 1989). Although it is not yet clear what biochemical contribution feeder cells make to protoplast cell wall biosynthesis, cell division, and callus differentiation, the use of feeder cells enhances protoplast growth. For this reason, feeder cells might be expected to improve the efficiency of antibiotic selection as well. Because of the interdependence of individual steps on the overall success of transformation, it is prudent to examine cell culture parameters for their direct impact on transformation efficiencies and gene expression.

Materials and methods

Cell Culture and Protoplast Isolation. Two lines of maize (*Zea mays* L.) A188 and Black Mexican Sweet (BMS) were crossed to make an F1 hybrid. An A188 x BMS cell suspension was initiated from a type II friable callus as described previously (Kamo *et al.* 1987). Suspension cultures were maintained on a gyratory shaker at 120 rpm, 25°C, in the dark. Subculturing was done every seven days by placing 5 ml packed (settled) cell volume (pcv) into 35 ml of N_6 medium (Chu *et al.* 1975) supplemented with 6 mM proline, 1 g/l casein hydrolysate, and 3.5 mg/L 2,4-D (Kamo *et al.* 1987). After 3 or 4 days the medium was removed from the flask and replaced with 35 ml of fresh medium. One week prior to protoplast isolation, 2 ml pcv of these suspension cells were subcultured into 41 ml of MS medium (Murashige and Skoog, 1962) supplemented with 3.5 mg/l 2,4-D. The protoplast isolation procedures were as described previously (Kamo *et al.* 1987). Protoplasts were resuspended in protoplast culture medium (PCM) at a density of 5 x 10^5 (or 10^6 in transformation experiments) viable protoplasts per ml, and 200 µl was plated onto 0.8 µm Millipore filters as described previously (Kamo *et al.* 1987).

Feeder Cells. Four different cell lines were evaluated for their effectiveness as feeder cells. Two of the lines were embryogenic suspension cultures of A188 x Black Mexican Sweet; one was grown in N_6 medium with 3.0 mg/l 2,4-D and the other line was grown in N_6 medium with 3.5 mg/l 2,4-D. The latter cell line was also used as the source of protoplasts. The other two cell lines were derived from a culture of nonembryogenic Black Mexican Sweet cultures that was provided by Dr. Ron Lundquist, Molecular Genetics, Inc. The nonembryogenic BMS cell lines were maintained in either MS medium with 0.01% (w/v) myo-inositol, modified White's vitamins (White, 1954) and 2.0 mg/l 2,4-D, oe N_6 medium with 3.0 mg/l 2,4-D. The feeder layer was prepared by adding PCM containing 0.2 M mannitol and 0.8% (w/v) LMP agarose to the appropriate number of cells (0.38 to 1.5 ml pv per 10 ml) and then transferring five ml to each Petri plate (15 x 60 mm).

Plasmid Constructions and Probes. The plasmid pPUR (5.68kb) was derived from pGEM and contained the 3.0 kb *Hin*dIII-*Eco*RI fragment of pBI121 (Jefferson *et al.* 1987). This restriction fragment consisted of the *uidA* coding region [which codes for ß-glucuronidase (GUS)] under the control of a CaMV 35S promoter and with a *nos* polyA tail (Fig. 1a). This construction was provided by Dr. Brian Larkins (Dept. of Botany and Plant Pathology, Purdue University). For Southern blotting, a ^{32}P-labeled probe was made from the *Bam*HI and *Sst*I restriction fragment of pPUR plasmid (1870 bp fragment; Fig. 1a). A 5.78kb pIC-19R-35S/Kan plasmid (obtained from Dr. Don Merlo, Lubrizol Genetics, Inc., Madison, WI and herein called pKAN) contained the *neo* coding region under the control of a CaMV 35S promoter with a T-DNA (orf 26) polyA tail. A ^{32}P-labeled probe was made from the 2.2 kb *Bam*HI restriction fragment (Fig. 1b).

Protoplast transformation and GUS assay. Transformation of protoplasts with pPUR or pKAN plasmids was facilitated by either PEG or electroporation. The PEG-mediated transformation was according to Antonelli *et al.* (1988) except that 1 x 10^7 protoplasts per 2 ml incubation mixture [1 ml protoplast suspension in 0.3 M mannitol and 80 mM $CaCl_2$ plus 1 ml 50% PEG in Krens' medium (Krens *et al.* 1982) were used, sonicated calf thymus DNA was omitted, and protoplasts were floated on top of 9% Ficol (in PCM) prior to and after the PEG treatment. Electroporation of A188 x BMS protoplasts was essentially as described by Kamo *et al.* (1987). Protoplasts ($3x10^6$) were resuspended in 1 ml of 4 mM Hepes, 60 mM NaCl, 1.6 mM $CaCl_2$, 0.3 M mannitol, pH 7.2. Fifty µg of pKAN DNA was added and the mixture was incubated in a 1 cm^2 plastic cuvette on ice for 5 min, and then electroporated with a model ZA 2010 electroporator (Prototupe Design Services, Madison Wisconsis). The settings were: 350V, 600µF and 100 mS. Following electroporation the sample was incubated at room temperature for 10 min and diluted with 2 ml of PCM every 5 min for 25 min. After centrifugation at 50xg for 10 min, protoplasts were resuspended in PCM and cultured on feeder cell layers as described above.

The assay for GUS activity and histochemical staining were performed according to the protocol of Jefferson *et al.* (1987) using 4-methylumbelliferyl-ß-D-glucronide and 5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid as the substrates, respectively. Protein was determined by the method of Lowry *et al.* (1951).

DNA Isolation and Analysis. DNA was extracted from maize callus by the method of Dellaporta *et al.* (1983). Digestion of the DNA with restriction enzymes, electrophoresis of the DNA fragments, and the Southern blotting procedures were performed essentially as described in Maniatis *et al.* (1982).

Chemicals. Chemicals were obtained from the following sources: abscisic acid (890-1010G) and coconut water (5700-06751 M) (Gibco Laboratories, Grand Island, NY); Bacto agar (Difco Co., Detroit, Mich, USA); restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, MD), agarose (5510UA) and low melting point (LMP) agarose (5517) (Bethesda Research Laboratories, Gaithersburg, MD); Calcofluor White (Polysciences, Warrington, PA); cellulase (LS02604) and pectinase (LS04298) (Cooper Biomedical , Cel, Malvern, PA); 2,4-D (D2128, Sigma Chemical Co. or D7,070-8, Aldrich Chemical Co., Milwaukee, WI); Murashige and Skoog basal salts (MM 100-5, Hazelton Research Products, Lenexa, KA). All other chemicals were from Sigma Chemical Co., St. Louis, MO.

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Results

Because of the low frequency and the random nature of gene insertions into the genome of plant protoplasts, it is important to develop culture conditions that result in high protoplast plating efficiencies. The A188 x BMS suspension cultures were subcultured from the maintenance N_6 medium to MS medium at 4 to 8 days prior to protoplast isolation. This medium change dramatically increased protoplast yields; cells grown in N_6 medium produced approximately 10^4 viable protoplasts per gFW while those transferred to MS medium produced at least 10^6 viable protoplasts per gFW. For these protoplasts to divide and grow into callus, it was essential that feeder cells be used (Table 1).

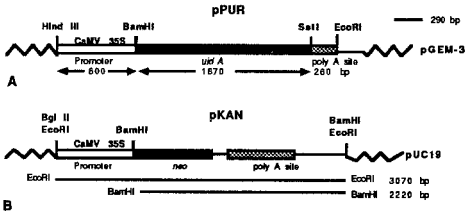

Fig. 1a. The pPUR plasmid was constructed by inserting the 3.0 kb *Hind*III-*Eco*RI fragment of pBI121 (Jefferson *et al.* 1987) into the polylinker of pGEM-3 (obtained from Promega, Inc.). pPUR contains the *uidA* gene coding region under the control of a CaMV 35S promoter. **b.** The pKAN plasmid has the *neo* gene under the control of a CaMV 35S promoter in pUC19. The probe used was the 2220 bp *Bam*HI fragment.

Table 1. Effect of feeder cells on callus growth from A188 x BMS protoplasts. Protoplasts were isolated from embryogenic A188 x BMS suspension cells and resuspended in liquid PCM. The protoplasts were plated onto Millipore filters over PCM medium solidified with LMP agarose and containing the indicated feeder cells at a density of 0.75 ml pcv / 10 ml. NE, non-embryogenic; E, embryogenic. Protoplast-callus growth was for one month.

Feeder Cells	Suspension Cell Medium Basal Salts	FW Callus/ Plate mg ± SE
None	-	0
NE BMS	MS	0
NE BMS	N_6	0
E A188 x BMS	MS	240 ± 120
E A188 x BMS	N_6	550 ± 210

Of the feeder lines tested, only the embryogenic A188 x BMS suspension cells served as an effective feeder layer for the A188 x BMS protoplasts (Table 1). In separate experiments, however, a small amount of callus growth from protoplasts could be obtained when using only BMS as feeders. The density of feeder cells also influenced protoplast growth (Table 2).

Table 2. Effect of feeder cell density on callus growth from A188 x BMS protoplasts. Protoplast-callus growth was for one month.

FEEDER CELL DENSITY [ml cells (pcv) / 10 ml medium]	GROWTH OF PROTOPLASTS (mg FW Callus / Petri plate ± SE)
0	0
0.38	640 ± 240
0.75	240 ± 40
1.50	50 ± 20

Transient expression of GUS gene was used to evaluate the effectiveness of PEG-mediated transformation and electroporation. Electroporation resulted in lower GUS activities in transformed protoplasts than PEG treatment (Figs. 2 and 4). Although, specific GUS activities determined in separate experiments varied significantly (because of this, results from separate experiments could not be pooled), electroporation consistently produced about 30% GUS activity of that observed in PEG treated protoplasts.

Histochemical staining of protoplasts 3-4 days after transformation revealed 47±8 blue spots on plates containing 200,000 viable plated protoplasts. After electroporation, 16±5 blue spots were observed.

Since feeder cells improved protoplast growth, it was of interest to determine if feeder cells would also improve the transient expression of foreign genes introduced into the protoplasts. To address this, protoplasts were transformed with pPUR and were cultured in one ml of liquid PCM

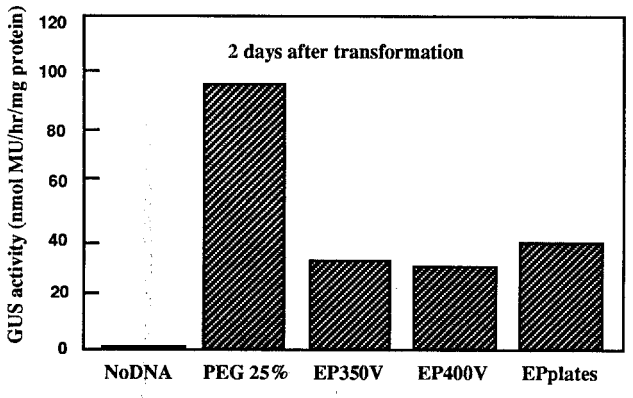

Fig. 2. Expression of GUS activity in protoplasts transformed by electroporation or PEG treatment. GUS activity was assayed 2 days after transformation in PEG-treated protoplasts without plasmid DNA (NoDNA) or with 50µg pPUR per incubation (PEG 25%). Electroporation (EP) was carried out with a single pulse of 350V or 400V using wire (EP 350V) or plate electrodes (EP plates)

medium at a density of 10^6 viable protoplasts per ml, or were plated on filters over agarose-solidified PCM medium with or without feeder cells.

Total GUS activity measured two days after transformation was 13-fold higher in cells cultured on agarose with feeder cells than those cultured in liquid PCM (Fig. 3). The feeder cell system alone (compare agarose, minus or plus feeder) increased total GUS activity by 5-fold. The differences in specific activity of GUS (nmole MU/hr/mg protein) were less pronounced. However, differences in specific activity were more evident five days after transformation (Fig. 4). Addition of conditioned medium (50% v/v) into liquid PCM increased specific GUS activity as compared to liquid PCM, but still it was twice as low as in protoplasts cultured on a feeder layer (Fig. 4).

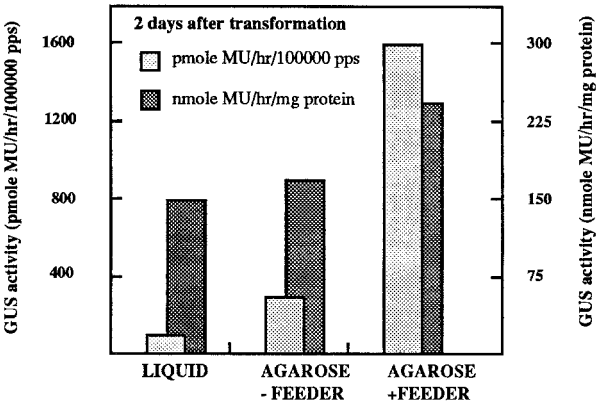

Fig. 3. The effect of protoplast culture conditions on transient expression of *uidA* gene in PEG-transformed maize protoplasts. Liquid PCM (LIQUID), agarose-solidified PCM without feeder cells (AGAROSE, - FEEDER), and with feeder cells (AGAROSE, + FEEDER) were used to culture the protoplasts after PEG-mediated transformation with pPUR. Protoplasts (pps) were incubated in liquid and on the solid support at a density of 10^6 viable protoplasts per ml.

To obtain stable transformation, maize protoplasts were also transformed with pKAN to confer kanamycin resistance. pKAN was transferred to protoplasts by PEG treatment and protoplasts were plated at a density of 10^6 per ml ($2x10^5$ protoplasts/plate). About 50% of the protoplasts survived the PEG treatment. The selection efficiency from PEG-mediated transformation was high -- about one transformant per 10^4 protoplasts (Table 3). Starting with 5-6 gFW of cells and using the protoplast - PEG transformation technique and the cell culture system with feeder cells, we routinely obtained approximately 500 lines of calli resistant to kanamycin.

A sample of 9 lines from 47 randomly-selected kanamycin-resistant calli tested for the presence of *neo* coding sequences in genomic DNA is presented in Fig. 5. If present, the *neo* gene was always in high molecular weight genomic DNA (Fig. 5b).

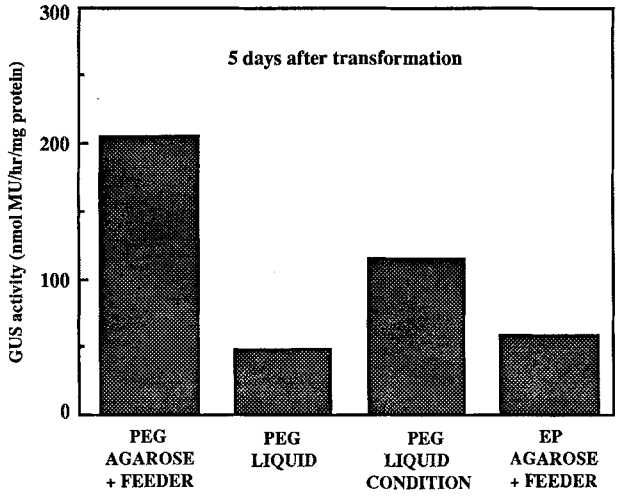

Fig. 4. The effect of protoplast culture conditions on GUS activity five days after transformation. Liquid PCM (LIQUID), PCM with conditioned medium (50% v/v) (LIQUID CONDITION), agarose-solidified PCM with feeder cells (AGAROSE+FEEDER) were used to culture the protoplasts after PEG-mediated transformation (PEG) or electroporation (EP). All other parameters as described in Materials and methods.

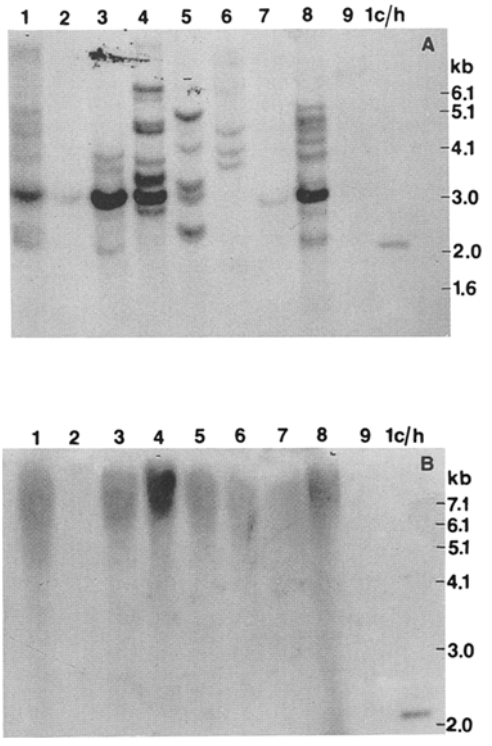

Fig. 5. Southern blot analysis of DNA isolated from kanamycin-resistant calli transformed with pKAN with the aid of PEG. Twenty µg of nucleic acid was loaded into each lane. The DNA samples were either digested with *Eco*RI (a) or were unrestricted (b). Both (a) and (b) were probed with ^{32}P-labeled *Bam*HI fragment of pKAN. The expected size of *Eco*RI and *Bam*HI fragments was 3.1 kb and 2.2 kb, respectively (Fig. 1). The *Bam*HI fragment of pKAN was used for reconstruction, indicating one copy per haploid genome (lane 1c/h). The callus for DNA analysis was randomly chosen among all kanamycin-resistant cell lines. Kanamycin selection procedure was the same as in Table 3.

Fig. 5a. shows the *Eco*RI restriction fragments of DNA isolated from these same 9 lines probed with the *Bam*HI fragment of pKAN. A variety of integration events was observed. Some callus lines (lanes 2 and 7; Fig. 5a) showed only one band hybridizing to the probe, suggesting that the intact *neo* coding region and poly A tail was present. Other cell lines (lanes 1,3,4,5,6, and 8; Fig. 5a) displayed multiple band patterns implying rearrangements had occurred during integration of the gene into the genome. One of them (lane 9) was apparently not transformed. Of the original 47 kan-resistant lines tested for the presence of the *neo* gene, 41 had a positive signal when probed with the *Bam*HI fragment (data not shown). Hence, 87% of the selected callus lines were stably transformed with the *neo* gene.

The pKAN plasmid was also introduced by electroporation, and in six separate experiments 58 callus lines were selected for kanamycin resistance. However, since protoplast growth after electroporation was slower then after PEG treatment, kanamycin selection was applied two weeks after transformation instead of at one week. Southern blot analysis of *Eco*RI restricted DNA from these 58 lines showed that five had positive signals when probed with the ^{32}P-labeled *Bam*HI fragment of pKAN (data not shown). These data indicated that only 9% of the selected callus lines had been transformed by the electroporation technique.

Table 3. Selection of kanamycin-resistant callus after PEG-mediated transformation with pKAN plasmid containing *neo* gene. After one week of culture on feeder cells at a density of 0.6 ml pcv / 10 ml, protoplasts were transferred onto fresh feeder cells supplemented with 100 µg/ml kanamycin sulfate. After an additional week, microcalli were transferred again onto fresh plates but feeder cells were omitted and PCM was replaced with MS media containing 100 µg/ml kanamycin sulfate. Two weeks later, callus colonies were selected, counted, and transferred to fresh plates containing MS (+2 mg/l 2,4-D) media with 100 µg/ml kanamycin sulfate. Protoplasts were plated at a density 1x10^6 protoplasts / ml (0.2 ml / plate). pKAN plasmid alone was used in experiment 1, whereas in experiments 2 and 3 pKAN and pPUR were both added before protoplast incubation (50 µg each).

Exp.	No. of plates used for selection	Total No. of selected colonies	No. of colonies/ plate (±SD)	Selection efficiency (No. selected colonies/ viable protoplasts)
1	12	223	18±3	1.0 x 10^{-4}
2	9	146	16±4	0.8 x 10^{-4}
3	8	220	27±4	1.3 x 10^{-4}

Discussion

Protoplast yields were increased markedly by subculturing the A188 x BMS suspension cells from N_6 medium into MS medium prior to protoplast isolation. This was observed with both maize and rice (Imbrie and Hodges 1986; Lee *et al.* 1989). For optimal protoplast growth into calli, feeder cells were required. The feeder effect was genotype specific with the embryogenic A188 x BMS cells supporting growth much better than the non-embryogenic BMS cells.

In our system, PEG treatment was a more reliable and effective method of introducing foreign genes into protoplasts than electroporation. It was not only advantagous for stable transformation as reported by Vasil *et al.* (1988) but also for the transient expression of introduced *uidA* genes. Since protoplasts were cultured in the same conditions, we conclude that fewer number of protoplasts have been transformed or survived electroporation as compared to PEG treatment. This conclusion was supported by histochemical staining of protoplasts after transformation.

The feeder cells had a profound effect on transient expression of GUS after PEG transformation. The presence of feeder cells in an agarose-solidified system increased the level of GUS activity more than 13-fold as compared to protoplasts cultured in liquid PCM medium, and by 5-fold when compared to protoplasts in agarose without feeders (Fig. 3). The difference in specific activity of GUS between these treatments was less pronounced at two days than five days after transformation. These results indicate that the Millipore filter/agarose/feeder system not only provides a better environment for protoplast survival after transformation but also affects gene expression.

The feeder cells also seem to help in the selection of stable transformants. Efficiency of selection was 10 to 100 times higher than generally reported for other transformation systems (Callis *et al.* 1987; Hauptmann *et al.* 1988). Timing and strength of selection is known to be crucial for efficient recovery of transformed callus (Christou *et al.* 1987). Since feeder cells support enhanced growth rates of protoplast cultures, they may allow a more rigorous selection for transformed cells and, thus, result in a decreased number of "escapes." In fact, our electroporation experiments not only produced fewer kanamycin-resistant calli as compared to PEG treatment but also increased the number of "escapes". This is probably related to the delay in the start of

kanamycin selection caused by reduced rate of growth of electroporated protoplasts. When the same selection strategy was applied after PEG treatment (100 µg/ml kanamycin sulfate applied two weeks after transformation), many "escapes" made efficient selection of transformed calli practically impossible.

Our PEG-mediated transformation procedure generally produced complex pattern of foreign gene insertions (Figs. 5a and b). Such patterns have been observed previously in other plant systems (Christou *et al.* 1987; Fromm *et al.* 1986; Krens *et al.* 1982) as well as in animal systems (Folger *et al.* 1982). The common occurrence of rearrangments and many copies of the foreign gene in transformants may be related to the concentration of DNA used and possibly to the application of high concentrations of kanamycin very early after transformation (Nellen and Firtel 1985).

In conclusion, the maize cell culture system that is described in this paper, coupled with PEG-mediated transformation, allows for the selection of several hundred stably transformed cell lines from a single experiment. Feeder cells markedly enhanced protoplast growth and division, and they also significantly improved transient gene expression and the efficiency of selection of genetically stable transformants. Since a rigorous selection scheme is often the bottleneck in plant genetic engineering (Hauptman *et al.* 1988), these results underscore the importance of cell culture parameters on efficient cell transformation.

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